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(54) Title: POLYPEPTIDES WITH TYPE IV COLLAGEN ACTIVITY		
(57) Abstract A composition which can bind heparin and promote cellular adhesion is provided which consists essentially of a polypeptide of the formula: met-phe-lys-lys-pro-thr-pro-ser-thr-leu-lys-ala-gly-glu-leu-arg, thr-ala-gly-ser-cys-leu-arg-lys-phe-ser-thr-met, asn-pro-leu-cys-pro-pro-gly-thr-lys-ile-leu, or mixtures thereof. Medical devices such as prosthetic implants, percutaneous devices, bandages and cell culture substrates coated with the polypeptide composition are also provided.		

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POLYPEPTIDES WITH TYPE IV COLLAGEN ACTIVITYGovernment Support

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Health. The Government has certain rights in the
invention.

Background of the Invention

10 Type IV collagen is a distinctive glycoprotein
which occurs almost exclusively in basement membranes.
It differs from the other types of collagen, which are
found mainly in interstitial connective tissue, with
regard to several structural properties. See New
15 Trends in Basement Membrane Research, K. Kuehn et al.,
eds., Raven Press, NY (1982) at pages 59-67. It has a
molecular weight (MW) of about 500,000 and consists of
three polypeptide chains: two $\alpha 1$ (MW 185,000) chains
and one $\alpha 2$ (MW 170,000) chain. Type IV collagen has
20 two major domains: a large, globular, non-collagenous,
NC1 domain and another major triple-helical, collagen-
ous domain. The latter domain is often interrupted by
short, non-collagenous sequences. The amino acid
sequence of this collagenous domain is only partially
25 known; however, the sequence of both the $\alpha 1$ - and $\alpha 2$ -
chains of the non-collagenous NC1 domain is known. See
U. Schwartz-Magdolen et al., FEBS Letters, 208, 203
(1986), and references cited therein. A diagrammatic
representation of the type IV collagen molecule is
30 shown in Figure 1. Apparently, type IV collagen is a
very complex and multidomain protein with different
biological activities residing in different domains.

Type IV collagen is an integral component of
basement membranes because it self-assembles to higher
35 forms which make up the supportive matrix of these
structures. Various other macromolecular components of
basement membranes are thought to assemble on this

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supportive framework. For example, laminin, nidogen and heparan sulfate proteoglycan have been reported to bind to type IV collagen. Laminin was observed to bind to two distinct sites along the length of the helix-rich, collagenous domain of type IV collagen. Nidogen and heparan sulfate proteoglycan were observed to bind specifically to the non-collagenous NCI domain.

Another property of type IV collagen is the ability to self-assemble by end-to-end and lateral associations, as mentioned hereinabove. The end-product of the polymerized structure is an irregular polygonal network. The NCI domain is required for network formation because it binds along the length of the helix-rich domain and brings adjacent molecules together, thus initiating lateral assembly. In the absence of lateral assembly, only end-to-end associations occur and the network-structure cannot be formed.

An additional function of type IV collagen is the binding to various cell types via cell surface receptors [M. Kurkinen et al., J. Biol. Chem., 259, 5915 (1984)]. M. Kurkinen et al. have reported that a major surface receptor protein with a molecular weight of 47,000 mediates this binding in the case of mouse embryo parietal endodermal cells.

The variety of functions attributed to type IV collagen indicates that this protein is an important reactant in many diverse and clinically important processes such as basement membrane assembly, cell migration, wound healing, tumor cell metastasis, diabetic microangiopathy, vascular hypertrophy due to hypertension and several kidney diseases. For example, Goodpasture's syndrome, a disease characterized by hemoptysis and hematuria due to alveolitis and nephritis, respectively, is associated with the presence of an antibody to the NCI domain of type IV collagen in

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the serum of all Goodpasture's patients. Another hereditary kidney disease, Alport's familial nephritis is apparently due to a genetic defect of the NC1 domain of type IV collagen. Finally, in diabetes mellitus,

5 intact type IV collagen, as well as the helix-rich domains, are chemically modified and functionally impaired by the increased amounts of glucose in the plasma and in the immediate vicinity of the basement membranes, i.e., in the extracellular matrix.

10 In order to better understand the pathophysiology of these processes at a molecular level, there is a need to try to assign each of the biological activities that type IV collagen exhibits to a specific sub-domain (i.e., NC1, helix-rich) or oligopeptide of type
15 IV collagen. If this can be achieved, it may be possible to synthesize small peptides which can provide the basis for important pharmaceutical compositions.

Brief Description of the Invention

20 The present invention provides three polypeptides which formally represent fragments of the α 1-NC1 chain of type IV collagen. The polypeptides, which can be prepared by conventional solid phase peptide synthesis, can be represented by the formulas:

25

met-phe-lys-lys-pro-thr-pro-ser-thr-leu-lys-ala-gly-glu-leu-arg (I),

thr-ala-gly-ser-cys-leu-arg-lys-phe-ser-thr-met (II),

30

and

asn-pro-leu-cys-pro-pro-gly-thr-lys-ile-leu (III).

35 Polypeptide I, (hereinafter "TS-1") formally represents amino acids 201-216 of the α 1-NC1 domain, and can be

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represented by the single letter code:

MFKKPTPSTLKAGELR. Polypeptide II formally represents isolated α 1-NC1 amino acid residues 49-50, while polypeptide III formally represents isolated α 1-NC1 amino acid residues 17-27. The single letter amino acid

codes for these polypeptides are TAGSCLRKFSTM and NPLCPPGTKIL, respectively. For brevity, polypeptide II will be hereinafter designated as "TS-2" and polypeptide III will be designated as "TS-3". Some of their properties, such as hydropathy index, net charge and number of lysines, are shown in Table I, below.

Table I

Peptide	Position in the α 1-NC1 Chain (a.a. No. from the NH ₂ -terminus)	Hydro- pathy* Index	Net Charge	Number of Lysine Residues
TS-1	201 - 216	-149	+3	3
TS-2	49 - 60	+11	+2	1
TS-3	17 - 27	-26	0	1

* Kyte and Doolittle, J. Mol. Biol., 157, 105 (1982).

These synthetic polypeptides were assayed for bioactivity and TS-2 and TS-3 were found to (a) bind to type IV collagen and to the isolated NC1 domain thereof (TS-2 only), (b) bind to heparin, and (c) to inhibit the binding of heparin to type IV collagen. TS-1, TS-2 and TS-3 promote the adhesion of aortic endothelial cells and bind to melanoma cells (TS-3 only). Therefore, it is believed that TS-1, TS-2 and TS-3 may be useful to (a) promote wound healing and implant acceptance, (b) promote cellular attachment to culture substrata and/or (c) inhibit the metastasis of malignant cells. Due to the difference in the spectra of biological activities

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exhibited by polypeptides TS-1, TS-2 and TS-3, mixtures of TS-1, TS-2 and TS-3 are also within the scope of the invention.

Furthermore, since it is expected that further
5 digestion/hydrolysis of polypeptides TS-2 and TS-3 in vitro or in vivo will yield fragments of substantially equivalent bioactivity, such lower molecular weight polypeptides are considered to be within the scope of the present invention.

10

Brief Description of the Figures

Figure 1 is a diagrammatic representation of the type IV collagen molecule. The length of the two triple helical segments is indicated on the top. Black
15 areas are those sites involved in the interaction with other type IV collagen molecules.

Figure 2 depicts the complete amino acid sequence of the $\alpha 1$ -NC1 chain of type IV collagen, in single letter amino acid code.

20 Figures 3A and 3B are graphical depictions of the effect of the polypeptides of the invention on type IV collagen network formation.

Figure 4 is a graphical depiction of the binding of intact NC1 domain to type IV collagen.

25 Figure 5 is a Scatchard plot of the binding of intact NC1 domain in solution to type IV collagen, which has been immobilized on a plastic surface.

Figure 6 is a graphical depiction of the binding of polypeptides TS-2 and TS-3 to intact NC1 domain
30 and to type IV collagen.

Figure 7 is a graphical depiction of the binding of the polypeptides of the invention to heparin.

Figures 8-9 are graphical depictions of the ability of polypeptides TS-2 and TS-3, respectively, to
35 bind to immobilized heparin.

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Figures 10-11 are graphical depictions of the binding of ^3H -heparin to type IV collagen and to NC1 domain, respectively.

Figures 12-13 are graphical depictions of the binding of increasing concentrations of ^3H -heparin to constant amounts of type IV collagen and NC1 domain, respectively.

Figures 14A-C are graphical depictions of the inhibition of ^3H -heparin binding to type IV collagen by polypeptides TS-1, TS-2 and TS-3, respectively.

Figure 15 is a graphical depiction of the binding of the present peptides to aortic endothelial cells.

Figure 16 is a graphical depiction of the binding of the present peptides to M₄ melanoma cells.

Figure 17 is a graphical depiction of the binding of the present peptides to normal rat fibroblasts.

Figure 18 is a graphical depiction of the binding of MM fibrosarcoma cells to peptides TS-1, TS-2 and TS-3.

Figure 19 is a graphical depiction of the binding of the present peptides to C6 glioma cells.

Figure 20 is a graphical depiction of the binding of peptides TS-1, TS-2 and TS-3 to A431 carcinoma cells.

Figure 21 is a composite depicting the binding of the present peptides to various cell lines.

Detailed Description of the Invention

The amino acid sequence of the helix-rich collagenous part of the $\alpha 1$ chain has been partially described by W. Babel et al., Eur. J. Biochem., **143**, 545 (1984). The full sequence of the noncollagenous $\alpha 1$ -NC1 is shown in Figure 2. The sequence information

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available on the globular NC1 domain of the $\alpha 1$ chain was examined and the three polypeptides of the invention, designated TS-1, TS-2 and TS-3, were synthesized for further evaluation.

5

Synthesis of Polypeptides The polypeptides of the invention were synthesized using the Merrifield solid phase method. This is the method most commonly used for peptide synthesis, and it is extensively described by J. M. Stewart and J. D. Young in Solid Phase Peptide Synthesis, Pierce Chemical Company, pub., Rockford, IL (2d ed., 1984), the disclosure of which is incorporated by reference herein.

The Merrifield system of peptide synthesis uses a 1% crosslinked polystyrene resin functionalized with benzyl chloride groups. The halogens, when reacted with the salt of a protected amino acid will form an ester, linking it covalently to the resin. The benzyloxy-carbonyl (BOC) group is used to protect the free amino group of the amino acid. This protecting group is removed with 25% trifluoroacetic acid (TCA) in dichloromethane (DCM). The newly exposed amino group is converted to the free base by 10% triethylamine (TEA) in DCM. The next BOC-protected amino acid is then coupled to the free amine of the previous amino acid by the use of dicyclohexylcarbodiimide (DCC). Side chain functional groups of the amino acids are protected during synthesis by TFA stable benzyl derivatives. All of these repetitive reactions can be automated, and the peptides of the present invention were synthesized at the University of Minnesota Microchemical facility by the use of a Beckman System 990 Peptide synthesizer.

Following synthesis of a blocked polypeptide on the resin, the resin-bound polypeptide is treated

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with anhydrous hydrofluoric acid (HF) to cleave the benzyl ester linkage to the resin and thus to release the free polypeptide. The benzyl-derived side chain protecting groups are also removed by the HF treatment.

5 The polypeptide is then extracted from the resin, using 1.0 M acetic acid, followed by lyophilization of the extract.

Lyophilized crude polypeptides are purified by preparative high performance liquid chromatography

10 (HPLC) by reverse phase technique on a C-18 column. A typical elution gradient is 0% to 60% acetonitrile with 0.1% TFA in H₂O. Absorbance of the eluant is monitored at 220 nm, and fractions are collected and lyophilized.

Characterization of the purified polypeptide

15 is by amino acid analysis. The polypeptides are first hydrolyzed anaerobically for 24 hours at 110°C at 6 M HCl (constant boiling) or in 4 N methanesulfonic acid, when cysteine or tryptophane are present. The hydrolyzed amino acids are separated by ion exchange chromatography using a Beckman System 6300 amino acid

20 analyzer, using citrate buffers supplied by Beckman. Quantitation is by absorbance at 440 and 570 nm, and comparison with standard curves. The polypeptides may be further characterized by sequence determination.

25 This approach is especially useful for longer polypeptides, where amino acid composition data are inherently less informative. Sequence determination is carried out by sequential Edman degradation from the amino terminus, automated on a Model 470A gas-phase sequenator

30 (Applied Biosystems, Inc.), by the methodology of R. M. Hewick et al., J. Biol. Chem., 256, 7990 (1981).

The invention will be further described by reference to the following detailed examples.

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Example 1.Liquid Phase Collagen Binding Assay

The ability of these peptides to bind to type IV collagen and to the NC1 domain was evaluated by a variety of approaches. In the first place, network formation by prewarmed intact type IV collagen was examined via the technique of rotary shadowing at the electron microscopic level. Table II lists the permutations which were tested and statistically evaluated (by counting the number of fields which contained irregular polygonal networks).

Table II

	<u>Run</u>	<u>Material</u>
15	(1)	type IV collagen alone (control).
	(2)	type IV collagen and albumin (BSA; another control).
	(3)	type IV collagen and isolated NC1 domain (control NC1).*
20	(4)	type IV collagen and diabetically modified or glucosylated NC1 domain.
	(5)	type IV collagen and peptide TS-1.
	(6)	type IV collagen and peptide TS-2.
	(7)	type IV collagen and peptide TS-3.

* The NC1 domain was isolated by collagenase treatment of murine, EHS-derived, purified type IV collagen, as described by Tsilibary et al. (J. Cell. Biol., 103, 2467-2473 (1986)).

30

The comparative assembly data obtained from permutations 1-4 are shown in Figure 3A. The comparative assembly data obtained from permutations 5-7 are shown in Figure 3B, which also shows the results obtained using type IV collagen with control NC1 domain and with glucosylated NC1 domain.

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The data summarized on Tables 3A and 3B confirm that: (a) control NCI domain binds to type IV collagen and competes for network formation; and (b) diabetically modified or glucosylated NCI domain cannot bind to type IV collagen because it cannot compete for network formation. Specifically, it was observed that polypeptide TS-2 can mimic the effect of intact, control NCI domain, since it can effectively prevent network formation. The other two peptides, TS-1 and TS-3, do not have any effect on the assembly of type IV collagen (Figure 3B).

Example 2.

Solid Phase Collagen Binding Assay

The binding of the intact NCI domain and polypeptides TS-2 and TS-3 to type IV collagen was also examined by solid phase binding assays. Type IV collagen was coated onto a plastic surface and the binding of isolated NCI domain was examined by adding increasing concentrations of ^{125}I -labelled NCI domain (isolated from purified type IV collagen by treatment with collagenase). These data are shown in Figure 4. It was observed that increasing amounts of ^{125}I -NCI bound to type IV collagen as the concentration of NCI added was increased, until a plateau was reached, indicating that all available sites for binding to the NCI domain were occupied. This saturable binding of ^{125}I -NCI to type IV collagen was specific since it could be competed for by an excess of unlabelled NCI domain (Figure 4).

Analysis of the affinity of the binding of NCI domain to type IV collagen by a Scatchard plot indicated two classes of binding sites with two different binding constants were present (Figure 5). The two binding constants were 40 nM and 330 nM, respectively.

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Type IV collagen in PBS (10 µg/ml, 50 µl) was added to plastic wells and allowed to bind to the plastic surface by incubation overnight at 4°C. Subsequently, ¹²⁵I-labelled NC1 domain was allowed to interact with the immobilized type IV collagen in the presence of BSA, peptide TS-2 or peptide TS-3. When peptide TS-3 was added in solutions of ¹²⁵I-NC1, it did not have any effect on the subsequent binding of ¹²⁵I-NC1 to type IV collagen (Figure 6). The presence of peptide TS-2 in solutions of ¹²⁵I-NC1 caused a dramatic increase of the binding of ¹²⁵I-NC1 to type IV collagen until a plateau was reached (Figure 6). These data indicate that peptide TS-2 can bind to type IV collagen and also to the isolated NC1 domain. This double binding ability of the peptide would account for the observed increase in binding of the NC1 domain to type IV collagen when higher concentrations of peptide TS-2 are used.

Example 3.

Heparin Binding Assays

In order to test the ability of the present polypeptides to bind to heparin, a solution of 2 mg/ml of each polypeptide in 50 mM ammonium bicarbonate (pH 7.8) was prepared and then serially diluted 1:1 in the same buffer to produce concentrations from 2 mg/ml to 1 µg/ml. One hundred µl from each dilution were incubated with ³H-heparin (50,000 dpm/ml) for two hours at 37°C and the mixtures were then added to nitrocellulose-coated wells. The wells were then washed in 10 mM Tris-HCl pH 8.0 (4X, 2 min each time). The amount of ³H-heparin bound to each peptide at each dilution is shown in Figure 7. The results of this experiment indicate that peptides TS-2 and TS-3 bind to heparin (TS-2 to a greater extent than TS-3), whereas TS-1 does not interact with heparin.

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The ability of the present polypeptides to interact with heparin when coated on 96-well plastic plates was evaluated. Stock solutions of the polypeptides at a maximum concentration of 2 mg/ml were prepared and serially diluted in phosphate-buffered saline (PBS)+NaN₃, producing final concentrations from 2 mg/ml to 1 µg/ml. Fifty µl from each dilution were coated on the 96-well plates and left to dry overnight at 28°C. Then, the wells were treated for two hours with 2 mg/ml BSA in order to minimize nonspecific ligand binding. ³H-heparin was added (50,000 dpm/well) and the mixture incubated for two hours. After extensive washing, the ³H-heparin bound at each peptide concentration was removed with sodium dodecyl sulfate (SDS) and counted in a scintillation counter. The results shown in Figure 8 indicate that peptide TS-2 binds heparin strongly. Peptide TS-3 also binds heparin, but less extensively (Figure 9). Peptide TS-1 does not bind heparin above background (BSA) values.

It was then established that ³H-heparin binds both to intact, native type IV collagen and to the isolated NC1 domain. Figures 10 and 11 show the binding of a constant amount of ³H-heparin (85,000 dpm) to increasing concentrations of type IV collagen and the NC1 domain, respectively. A saturable binding is observed in both cases. Type IV collagen binds 5-6 times more ³H-heparin than does the NC1 domain.

Next, the binding of increasing concentrations of ³H-heparin to a constant amount of type IV collagen (3 µg) and an equimolar amount of the NC1 domain (1 µg) was tested. Again, a saturable binding of ³H-heparin to type IV collagen (Figure 12) and to the NC1 domain (Figure 13) was observed. In this type of experiment, approximately 4 times more ³H-heparin bound to type IV collagen than to the NC1 domain. These data indicate

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that at least two binding sites for heparin exist in type IV collagen: one in the NC1 domain and the other(s) in the helix-rich part of the molecule.

Next, solutions of the three peptides of the invention (not absorbed to plastic) were screened for ability to inhibit the binding of heparin to intact, native type IV collagen coated on plastic. This experimental approach is intended to obviate problems due to any differential coating of peptides in heparin binding assays.

Type IV collagen at 60 $\mu\text{g/ml}$ in PBS + 0.1% Triton-X was coated on 96-well plates (use of 50 μl or 3 μg of laminin per well), dried overnight at 28°C and then the wells were coated with 2 gm/ml bovine serum albumin (BSA) in PBS. Peptides at various dilutions ranging from 2 mg/ml to 1 $\mu\text{g/ml}$ were co-incubated with a standard amount of ^3H -heparin (30,000 dpm/well) for two hours and the mixture was then transferred to the type IV collagen-coated plates and allowed to incubate for another two hours. After extensive washing, the radioactivity retained in each well was counted.

The results shown in Figure 14 indicate that peptides TS-2 (Figure 14B) and TS-3 (Figure 14C) interact with heparin in this assay, since they both exhibited an about 43% inhibition of the binding of ^3H -heparin to collagen at the highest concentration tested. However, peptide TS-2 was able to significantly inhibit the binding of ^3H -heparin to type IV collagen at lower concentrations than TS-3, and therefore, bound to heparin with higher affinity. TS-1 does not interact with heparin by this assay (Figure 14A).

Peptides TS-2 and TS-3 were able to bind to heparin in all the assays which were performed. It is interesting that peptide TS-2 binds both to heparin and to type IV collagen. These data indicate the presence

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of more than one binding site in this peptide. Therefore, it is possible that binding of heparin to one binding site of this peptide competes for the binding of the peptide to type IV collagen. Also, this peptide
5 could be used to link heparin and type IV collagen together, as well as to bind them to various biomaterials.

Example 4.

10 Cell Adhesion Assays

These assays were performed with the following cell lines: aortic endothelial cells, metastatic melanoma murine (M₄) cells, normal rat fibroblasts, MM fibroscarcoma cells, C6 glioma cells and breast carcinoma (A431) cells. Cell binding assays were performed
15 in the same way for each cell line and the three peptides of the invention, TS-1, TS-2 and TS-3 were individually assayed in each case for cell adhesion.

Adhesion was tested in each case, using a 96-
20 well microtiter plates absorbed with four different amounts (0.5; 5; 50; and 500 µg/ml, 100 λ/well) of peptides TS-1, TS-2, TS-3 and BSA. Cultures of cells which were 60-80% confluent were metabolically labelled for 24 hours by the addition of 3 mCi of ³H-thymidine.
25 On the day of the assay, the cells were harvested by trypsinization, the trypsin was inhibited by the addition of serum and the cells were washed free of this mixture and resuspended in DMEM. The cells were adjusted to a concentration of 6×10^5 /ml and 100 µl of
30 this cell suspension was added to the wells. The assay mixture was then incubated at 37°C for 90 min. At the end of the incubation, the wells were washed with warm PBS containing 10 mM Ca⁺⁺ and the adherent population was solubilized with 0.5 M NaOH containing 1% sodium
35 dodecyl sulfate. The solubilized cells were then quantitated using a liquid scintillation counter. Each determination was done in triplicate.

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A. Aortic Endothelial Cells

Aortic endothelial cells were obtained from bovine aortas by treatment with collagenase and were frozen at -196°C until use. These cells were cultured in primary cultures in the presence of DMEM and 10% fetal calf serum at 37°C in a humid atmosphere. When aortic endothelial cells were about 70% confluent, they were released from the tissue culture plastic by trypsin and then were added in suspension to the wells of 96-well plates coated with the following peptides: (a) TS-1, (b) TS-2 and (c) TS-3, in the concentrations described above.

The cells were metabolically labelled with 3.0 mCi of ^3H -thymidine for 24 hours prior to the assay. After trypsinization, the cells were allowed to attach for a 90-minute incubation period in the peptide-coated wells. After extensive washing, the radioactivity associated with each well was measured and used as an index of cell attachment (expressed as percent adherent). The data summarized on Figure 15 shows that peptide TS-1 was the most potent in causing the adhesion of aortic endothelial cells, followed by peptides TS-2 and TS-3, respectively. In Figure 15, the background values due to BSA binding were subtracted.

B. Metastatic Melanoma Cells

Highly metastatic melanoma cells, K1735M4, were originally provided by Dr. I. J. Fidler of Houston, TX. When the cells were received, a large number of early passage cells were propagated and frozen in liquid nitrogen. The tumor cells are usually cultured in vitro for no longer than six weeks. Following this period, the cells are discarded and new cells withdrawn from storage for use in further in vitro or in vivo experiments. This precaution is taken

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to minimize phenotypic drift that can occur as a result of continuous in vitro passage. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% heat inactivated fetal calf serum. The
5 - cultures were grown in 37°C incubators under a humidified atmosphere containing 5% CO₂. Cells were subcultured twice weekly by releasing cells gently from the flask, using 0.05% trypsin and 1 mM EDTA.

The melanoma cells were metabolically labelled
10 in the same fashion as the endothelial cells described hereinabove, using 2 µCi/ml ³HTdR (tritiated thymidine). The labelled cells were harvested as described for the endothelial cells. The cell adhesion assay was identical to that described hereinabove for
15 the bovine aortic endothelial cell assay.

The data summarized in Figure 16 demonstrates that peptides TS-1, TS-2 and TS-3 promote adhesion of M₄ cells. Peptide TS-1 was the most potent in this respect. In this figure, adhesion of M₄ cells to
20 intact type IV collagen and isolated NC1 domain is shown for comparison. Background adhesion to BSA has been subtracted.

C. Normal Rat Fibroblasts

25 These cells were obtained from cultures of rat dermis explants in DMEM containing 10% fetal calf serum, in plastic wells. Under these conditions, fibroblasts migrated to the bottom of the plastic dish. When the cells were confluent, they were har-
30 vested by trypsinization and were then metabolically labelled, as described above, for the cell adhesion assay. Figure 17 shows that peptide TS-1 was the most potent in promoting adhesion of these cells followed by peptides TS-2 and TS-3, which promoted adhesion of rat
35 fibroblasts to a minimal extent. Background adhesion to BSA has been subtracted.

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D. Isolation of and Cell Adhesion Assay for MM Fibro-sarcoma Cell Line

Murine fibrosarcoma cells (uv-2237-MM) were originally provided by Dr. I. J. Fidler of Anderson
5 Hospital, University of Texas Health Sciences Center, Houston, TX. Culturing, labelling and harvesting techniques were as described in Part A. The results of this assay are summarized in Figure 18. Background adhesion to BSA has been subtracted.

10

E. Isolation of and Cell Adhesion Assay for C6 Cell Line

Rat C6 glioma cell line was purchased from the American Type Culture Collection (identification number
15 CCL 107). Culturing techniques were as described in Part A. Labelling and harvesting techniques were as described above, under Example 4. The results of this assay are summarized in Figure 19. Peptides TS-1, TS-2 and TS-3 promoted adhesion of C6 glioma cells. At the
20 highest concentration, peptide TS-1 was the most potent in promoting adhesion. Background adhesion to BSA has been subtracted.

F. A431 Breast Carcinoma Cells

25 A431 cells were purchased from the American Type Culture Collection. Culturing, labelling and harvesting techniques have been described above (see methodology under Example 4, and Part A). The results of this assay are summarized in Figure 20. Background
30 adhesion to BSA has been subtracted.

Figure 21 summarizes the data with respect to the adhesion of the above-mentioned peptides TS-1, TS-2 and TS-3 to the previously described cell lines. Two other irrelevant peptides were also used: JM-8, as a
35 positive control (a peptide derived from the sequence

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of fibronectin, which is known to promote cell adhesion) and F-11 (a peptide derived from the sequence of the β_1 chain of laminin, which does not promote cell adhesion and which was used as a negative control).

- 5 Adhesion to isolated domain NC1 is also shown for comparison. Background adhesion to BSA has been subtracted.

In summary, peptide TS-1 promotes adhesion of aortic endothelial cells, metastatic carcinoma M4
10 cells, normal rat fibroblasts, MM fibrosarcoma cells, C6 glioma cells and A431 breast carcinoma cells. Peptide TS-2 binds (a) to type IV collagen, (b) to heparin and (c) promotes adhesion of the above-mentioned cell lines. Peptide TS-3 (a) binds to
15 heparin and (b) promotes adhesion of the above-mentioned cell lines.

A number of practical applications for polypeptides TS-1, TS-2 and TS-3 can be envisioned. Such applications include the promotion of the healing of
20 wounds caused by the placement of natural or synthetic substrata within the body. Such synthetic substrata can include artificial vessels, intraocular contact lenses, hip replacement implants and the like, where cell adhesion is an important factor in the acceptance
25 of the synthetic implant by normal host tissue.

As described in U.S. Patent No. 4,578,079, medical devices can be designed making use of these polypeptides to attract cells to the surface in vivo or even to promote the growing of a desired cell type on a
30 particular surface prior to grafting. An example of such an approach is the induction of endothelial cell growth on a prosthetic device such as a blood vessel or vascular graft, which is generally woven or knitted from a synthetic resin such as nitrocellulose, expanded
35 polytetrafluoroethylene or polyester fiber, particularly DacronTM (polyethylene terephthalate) fiber.

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Devices intended for cardiac insertion include temporary left ventricular assist devices, heart valves, intraortic balloon pumps and artificial hearts. Such devices are preferably formed from synthetic resins
5 such as polyether-type polyurethane elastomers (CardiothaneTM, Kontron) or from vulcanized polyolefin rubbers (HexsynTM, Goodyear).

Most types of cells are attracted to collagen and to the present polypeptides, but endothelial cells,
10 epithelial cells and fibroblastic cells in particular may be strongly attracted to the present polypeptides. The latter point indicates the potential usefulness of these defined polypeptides in coating a patch graft or the like for aiding wound closure and healing following
15 an accident or surgery.

In such cases, it may be advantageous to couple the peptide to a biological molecule, such as collagen, a glycosaminoglycan or a proteoglycan. Collagens, proteoglycans and glycosaminoglycans are
20 major components of connective tissues and basement membranes. In some cases, prosthetic devices formed entirely or in part from naturally-occurring tissues instead of synthetic polymers are used. One example is the use of porcine heart valves to replace defective
25 human heart valves. Such artificial valves can also comprise human dura matter or bovine pericardium. Another example is the use of bovine arteries as vascular grafts.

It may be useful to coat surfaces of these
30 biological substrata with the present polypeptides, in order to modify the cellular response, in vivo, thus improving the therapeutic outcome. This can be achieved by a variety of methods known to the art, e.g., by direct binding of the polypeptides to the
35 target surfaces based on the affinities described hereinabove, or by the covalently bonding the polypeptides

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to the substrate using various crosslinking reactions or reagents. For a review of the use of synthetic resins and biomaterials in prosthetic devices, see Chem. & Eng. News (April 14, 1986) at pages 30-48, the disclosure of which is incorporated by reference herein.

It is also indicative of their value in coating surfaces of a prosthetic device which is intended to serve as a temporary or semipermanent entry into the body, e.g., into a blood vessel or into the peritoneal cavity, sometimes referred to as a percutaneous device. Such devices include controlled drug delivery reservoirs or infusion pumps.

Also, polypeptides TS-1, TS-2 and TS-3 can be used to promote endothelial, fibroblast or epithelial cell adhesion to naturally occurring or artificial substrata intended for use in vitro. For example, a culture substrate such as the wells of a microtiter plate or the medium-contacting surface of microporous fibers or beads, can be coated with the cell-attachment polypeptides. This can obviate the use of fibronectin in the medium, thus providing better defined conditions for the culture as well as better reproducibility.

As one example of commercial use of cell-attachment surfaces, CytodexTM particles, manufactured by Pharmacia, are coated with gelatin, making it possible to grow the same number of adherent cells in a much smaller volume of medium than would be possible in dishes. The activity of these beads is generally dependent upon the use of fibronectin in the growth medium and the present polypeptides are expected to provide an improved, chemically-defined coating for such purposes. Other surfaces or materials may be coated to enhance attachment, such as glass, agarose, synthetic resins or long-chain polysaccharides.

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Finally, TS-1, TS-2 and TS-3 can be used to coat the surface of medical devices intended for external application or attachment to the body. Such devices include "bandages", which term is also intended to refer to wound packs and dressings, which can comprise surfaces formed from absorbent cellulosic fibers, from synthetic fibers or from mixtures thereof. These surfaces can be coated with amounts of TS-1, TS-2 and/or TS-3 effective to promote cellular growth, wound healing, graft attachment and the like.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

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WHAT IS CLAIMED IS:

1. A composition consisting essentially of a polypeptide of the formula:

met-phe-lys-lys-pro-thr-pro-ser-thr-leu-lys-
ala-gly-glu-leu-arg,

thr-ala-gly-ser-cys-leu-arg-lys-phe-ser-thr
met,

asn-pro-leu-cys-pro-pro-gly-thr-lys-ile-leu,

or mixtures thereof.

2. A prosthetic device designed for placement in vivo, comprising a surface coated with a composition consisting essentially of a polypeptide of the formula:

met-phe-lys-lys-pro-thr-pro-ser-thr-leu-lys-
ala-gly-glu-leu-arg,

thr-ala-gly-ser-cys-leu-arg-lys-phe-ser-thr
met,

asn-pro-leu-cys-pro-pro-gly-thr-lys-ile-leu,

or mixtures thereof.

3. The prosthetic device of claim 2, wherein said surface constitutes a portion of a vascular graft.
4. The prosthetic device of claim 2, wherein said surface constitutes a portion of an intraocular contact lens.

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5. The prosthetic device of claim 2, wherein said surface constitutes a portion of a heart valve.
6. The prosthetic device of claim 2, wherein said surface constitutes a portion of a hip replacement implant.
7. The prosthetic device of claim 2, wherein said surface constitutes a portion of a percutaneous device.
8. The prosthetic device of claim 2 wherein said surface is made of a synthetic resin.
9. The prosthetic device of claim 8 wherein said synthetic resin is selected from the group consisting of nitrocellulose, polyurethane, expanded polytetrafluoroethylene, polyester and polyolefin.
10. The prosthetic device of claim 2 wherein said surface is made of a naturally-occurring tissue.
11. A cell culture substrate having a surface coated with a composition consisting essentially of a polypeptide of the formula:

met-phe-lys-lys-pro-thr-pro-ser-thr-leu-lys-
ala-gly-glu-leu-arg,

thr-ala-gly-ser-cys-leu-arg-lys-phe-ser-thr
met,

asn-pro-leu-cys-pro-pro-gly-thr-lys-ile-leu,

or mixtures thereof.

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12. The cell culture substrate of claim 11 wherein said surface is made of a synthetic resin.
13. The cell culture substrate of claim 11 wherein said surface constitutes a portion of a bead.
14. The cell culture medium of claim 11 wherein said surface constitutes a portion of a microporous fiber.
15. The cell culture medium of claim 11 wherein said surface constitutes the wells of a microtiter plate.
16. A bandage comprising a fibrous surface coated with a composition consisting essentially of a polypeptide of the formula:

met-phe-lys-lys-pro-thr-pro-ser-thr-leu-lys-
ala-gly-glu-leu-arg,

thr-ala-gly-ser-cys-leu-arg-lys-phe-ser-thr
met,

asn-pro-leu-cys-pro-pro-gly-thr-lys-ile-leu,

or mixtures thereof.
17. The bandage of claim 16 wherein the fibrous surface comprises cellulosic fibers.

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FIG. 1

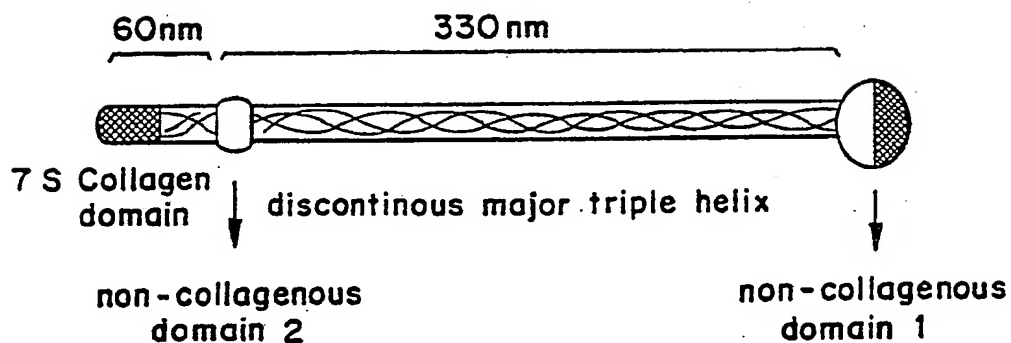


FIG. 2

10	20	30	40	50	60	70
SVDHGFLVTR	HSQTDDPLC	PPGTKILYGH	YSLLYVQGNE	RAHGQDLGTA	GSCLRKFSTM	PFLFCNINNV
80	90	100	110	120	130	140
CNFASTRNDYS	YWLSTPEPMP	MSMAPISGDN	IRPFISRCAY	CEAPAMVMAY	HSQTIQIPQC	PNGWSSLWIG
150	160	170	180	190	200	210
YSFVMHTSAG	AEGSGQALAS	PGSCLEEFRS	APFIECHGRG	TCNYANAYS	FWLATIERSE	MFKKPTPSTL
220						
KAGELRTHVS	RCQVCMRRT					

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FIG. 3A

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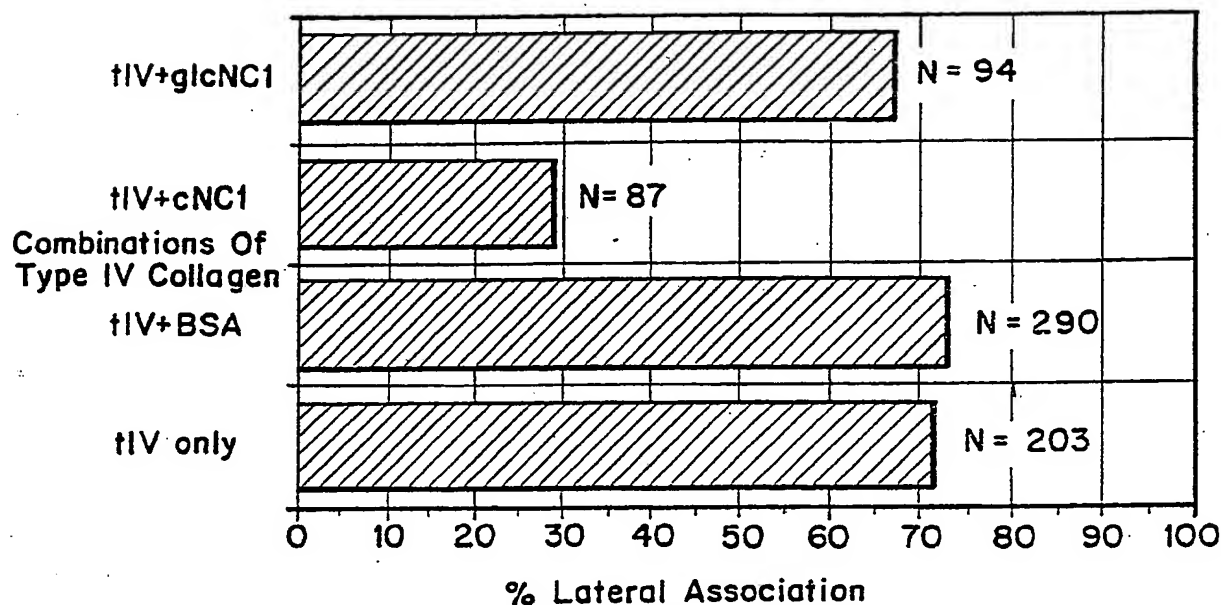
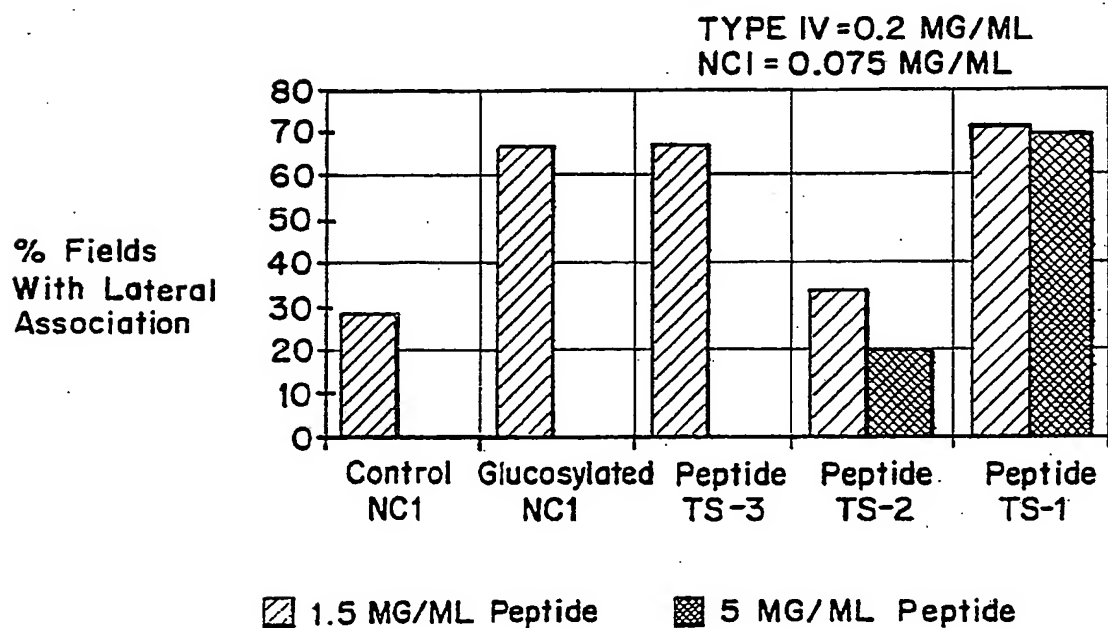


FIG. 3B



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FIG. 4

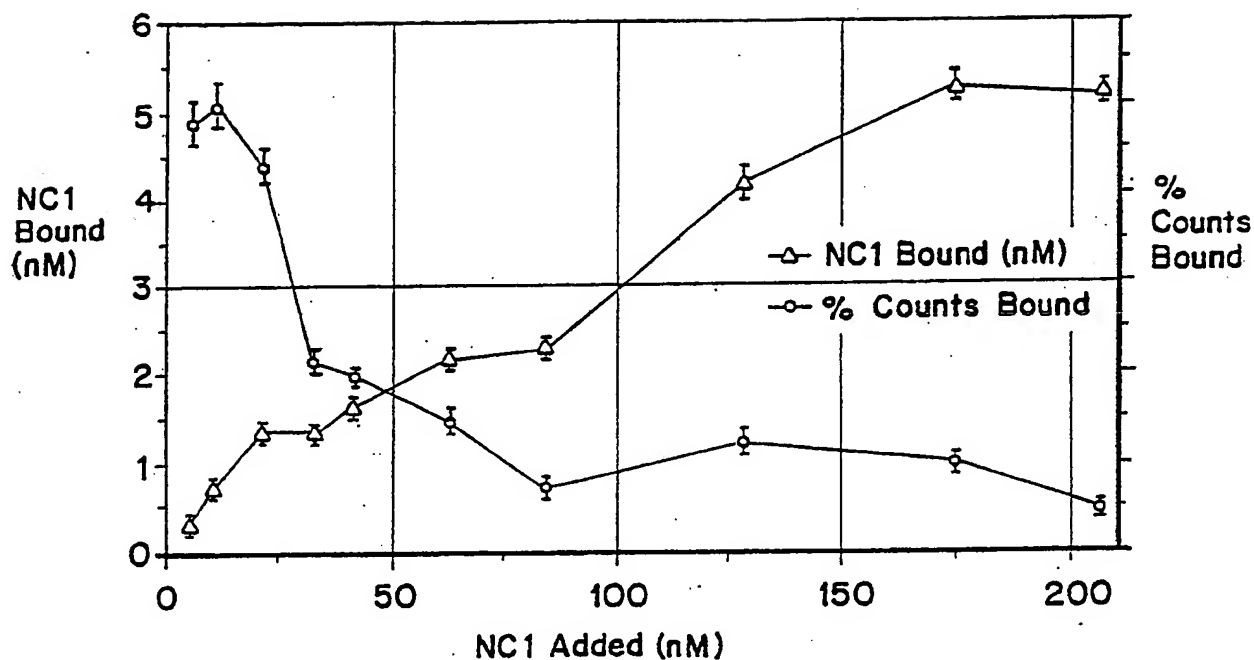
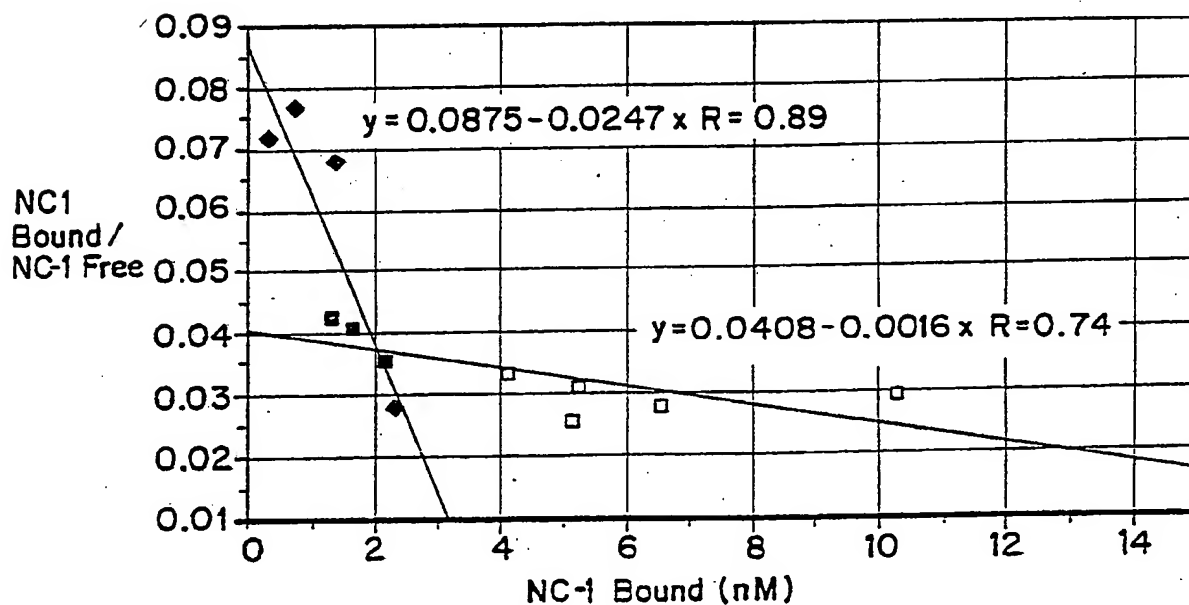


FIG. 5



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FIG. 6

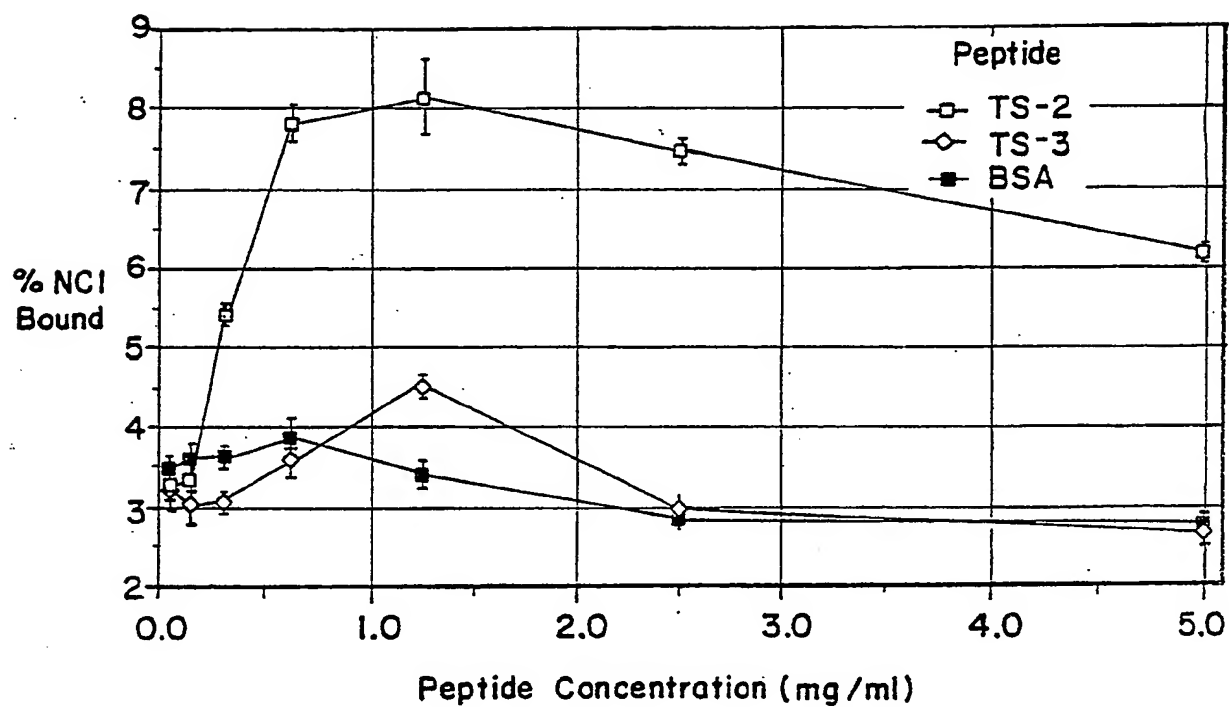
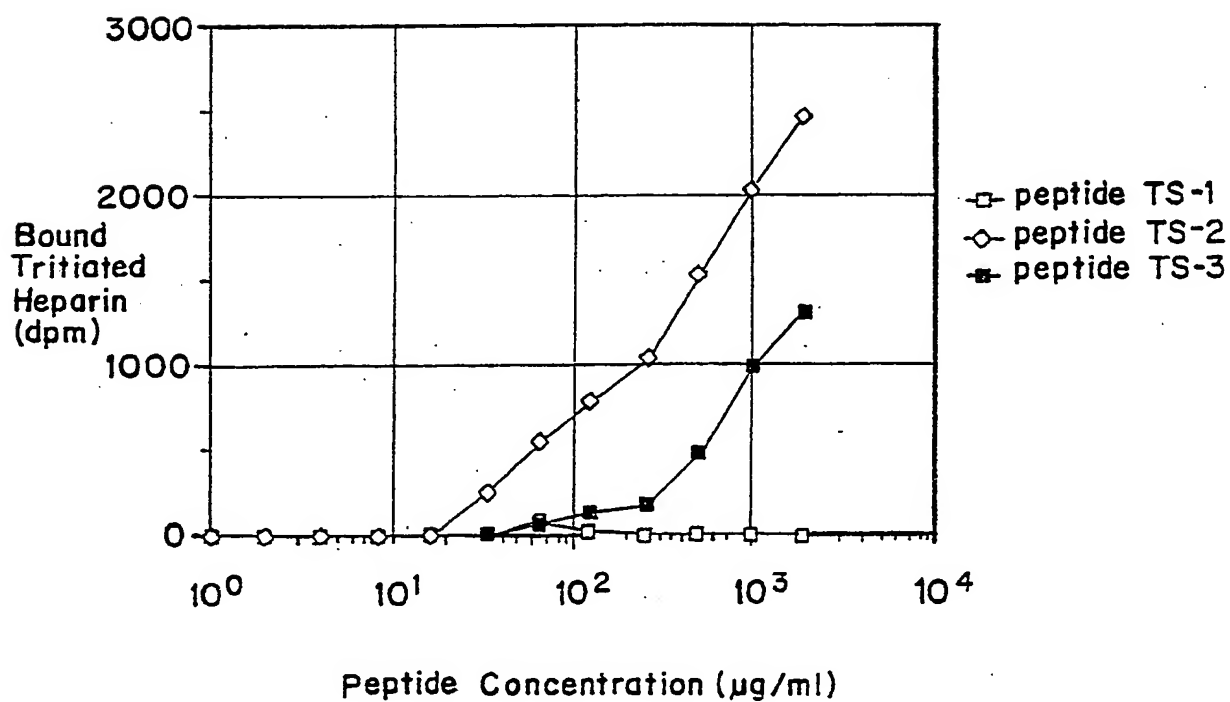


FIG. 7



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FIG. 8

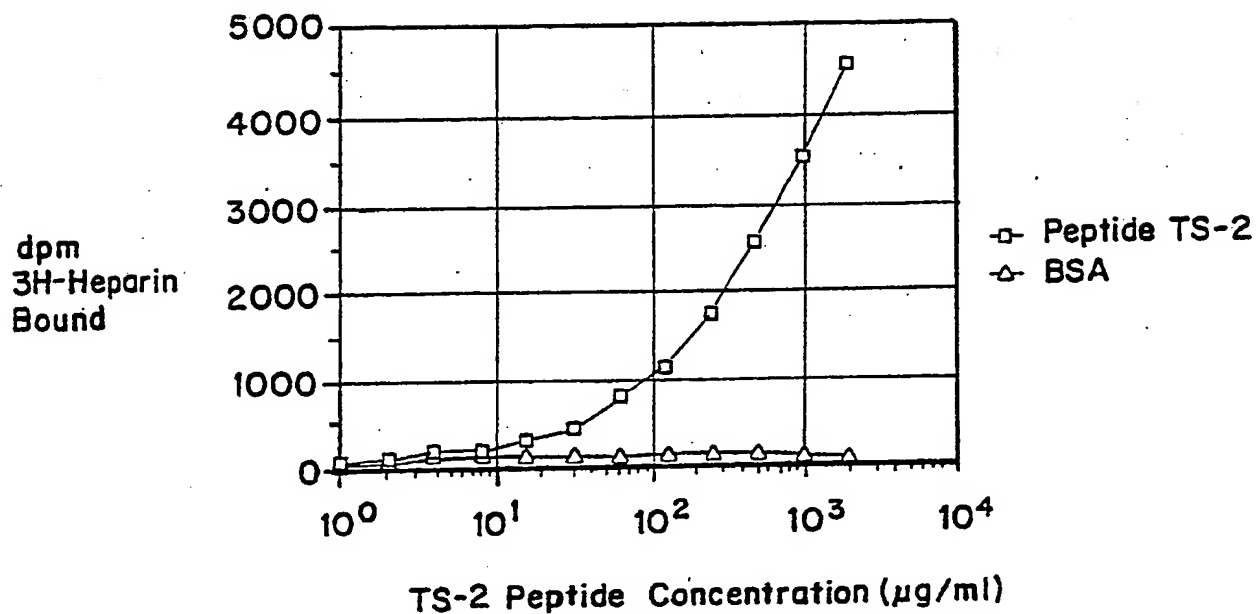
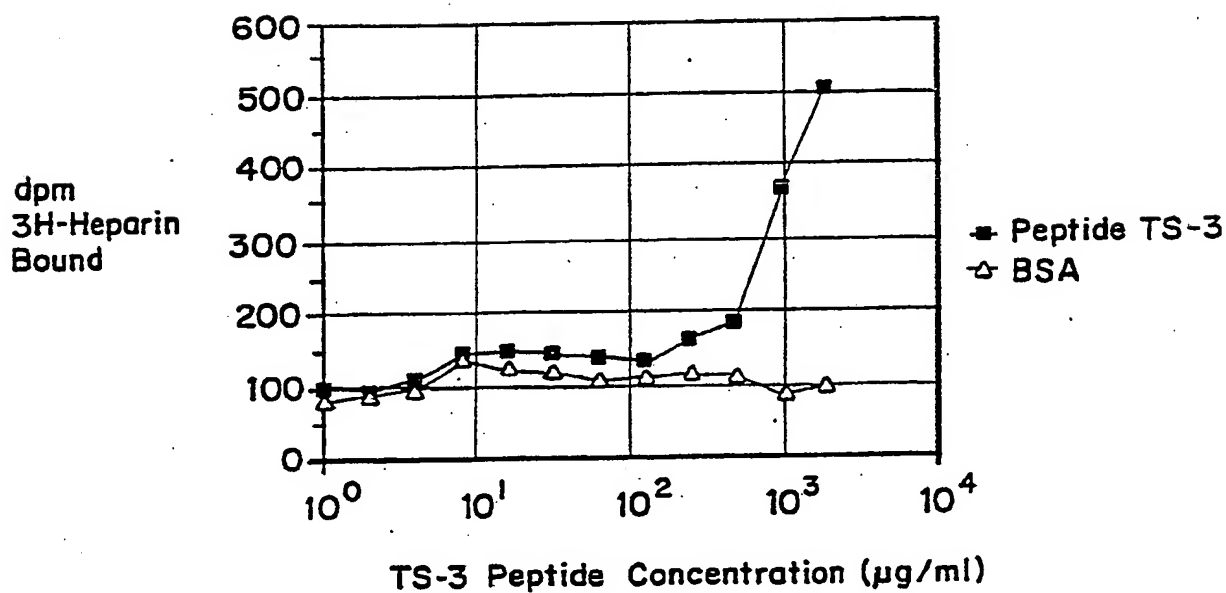


FIG. 9



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FIG. 10

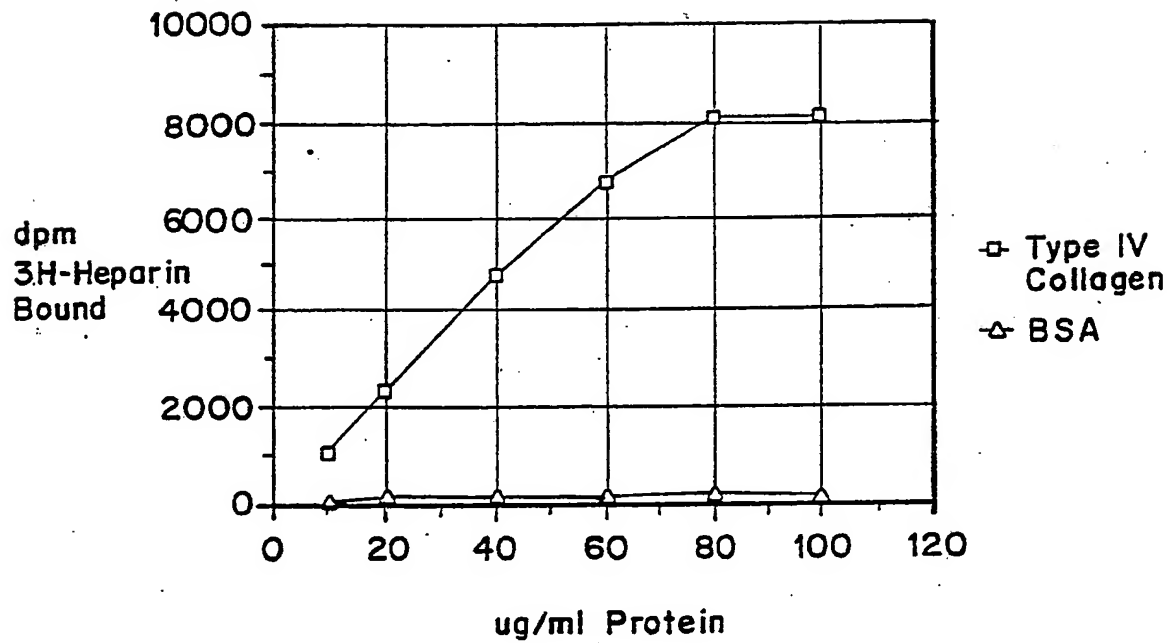
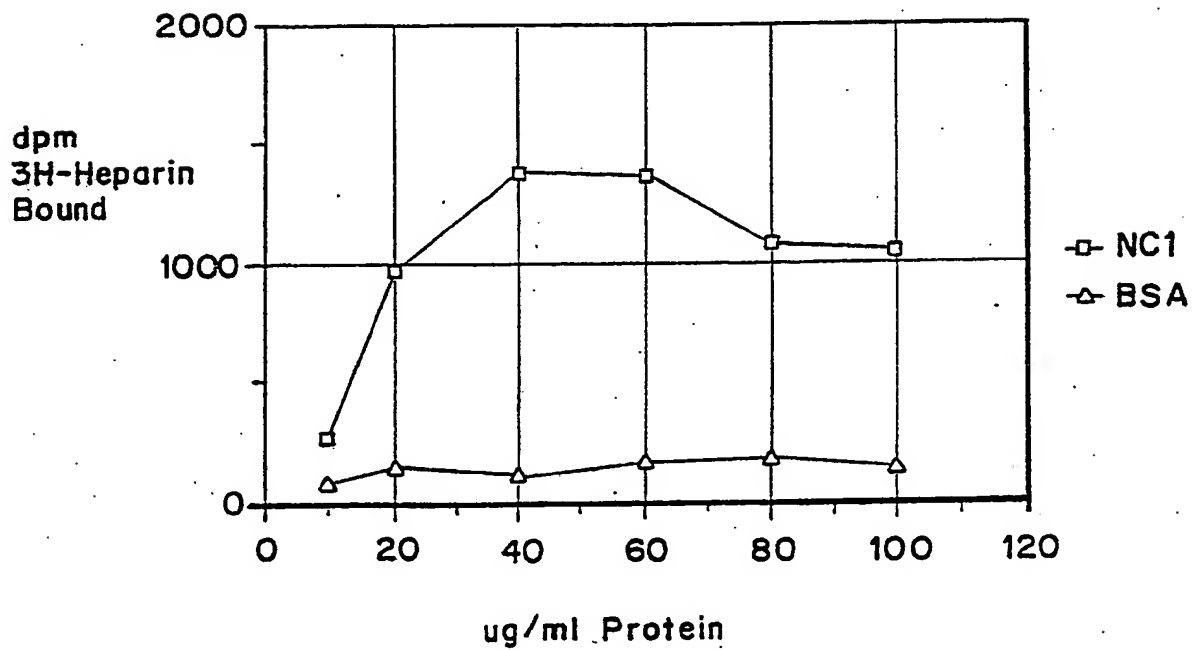


FIG. II



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FIG. 12

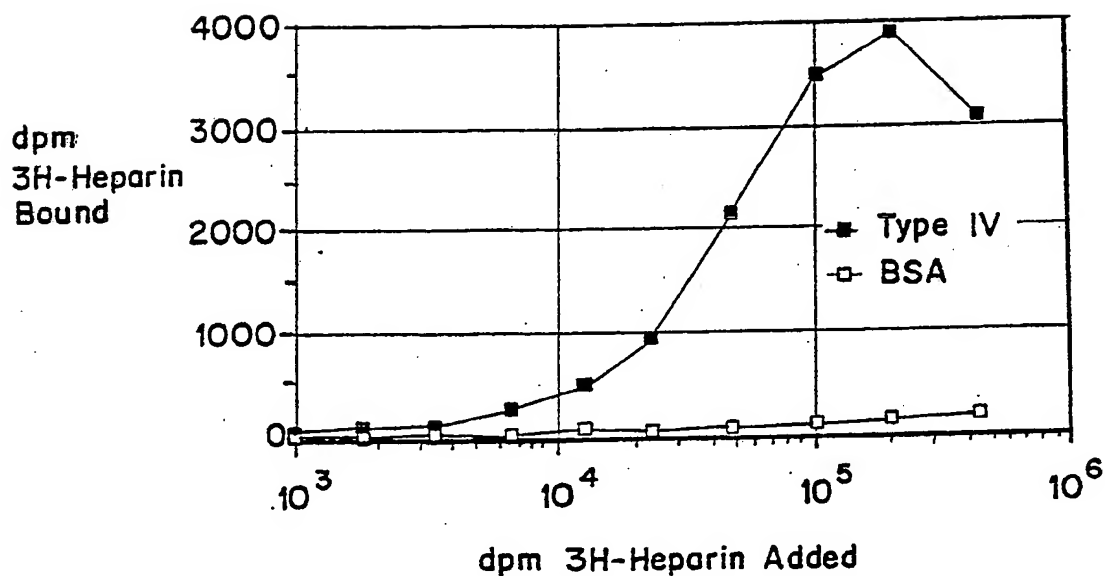
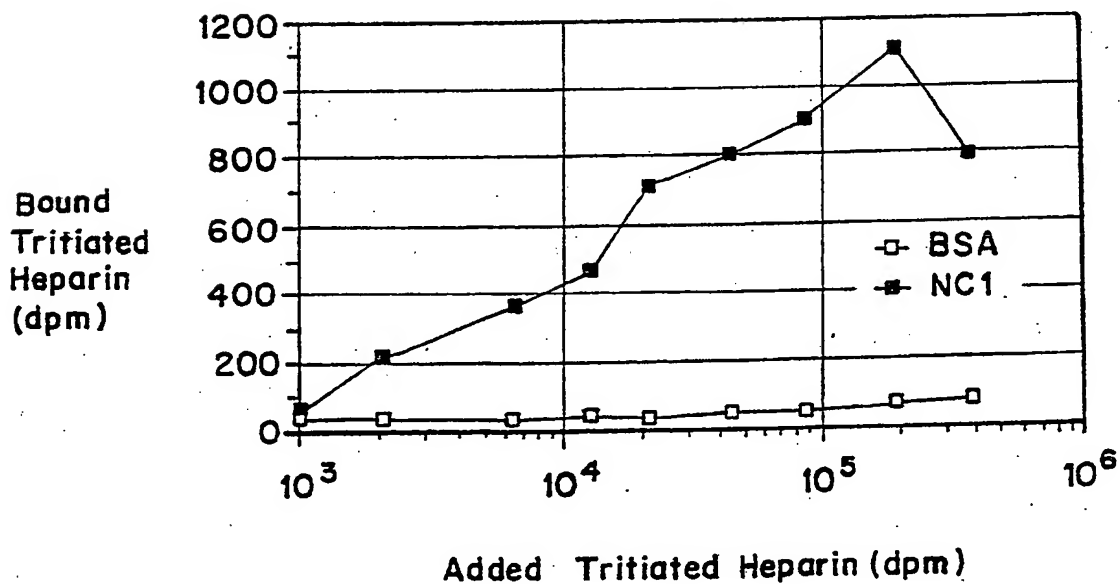


FIG. 13



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FIG. 14A

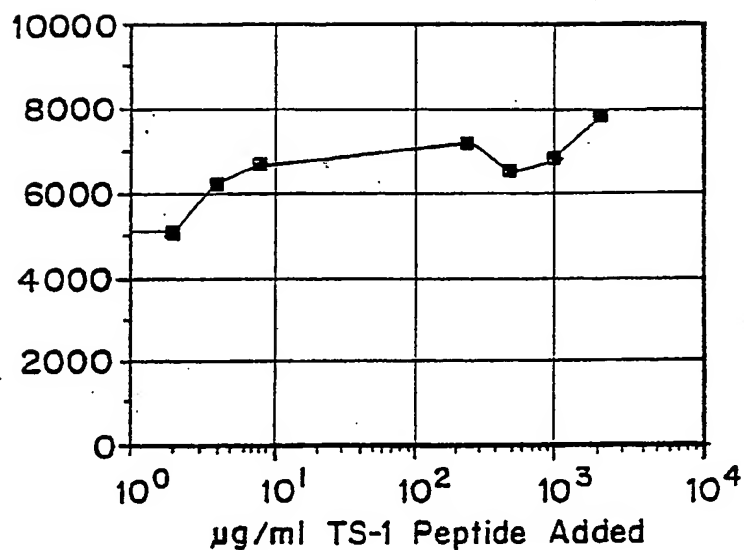
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3H-Heparin
Bound

FIG. 14B

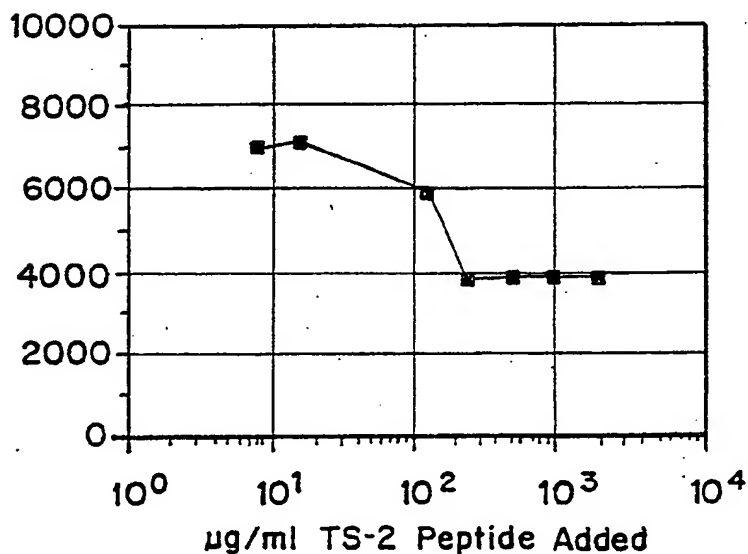
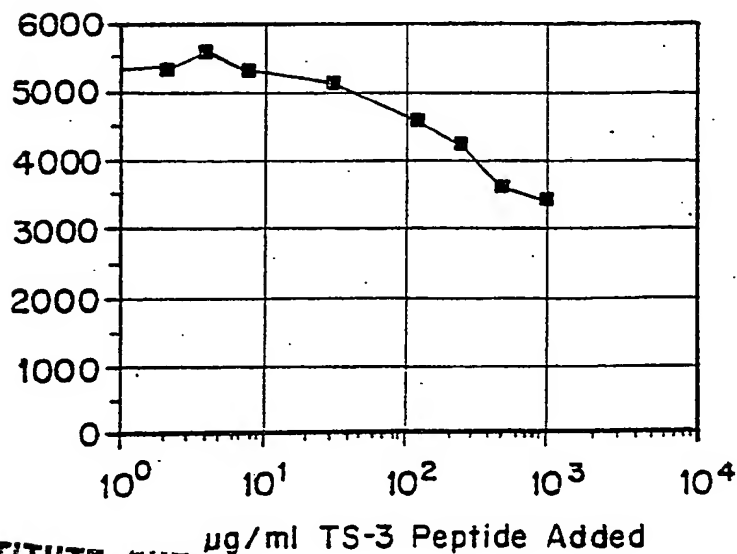
dpm
3H-Heparin
Bound

FIG. 14C

dpm
3H-Heparin
Bound

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FIG. 15

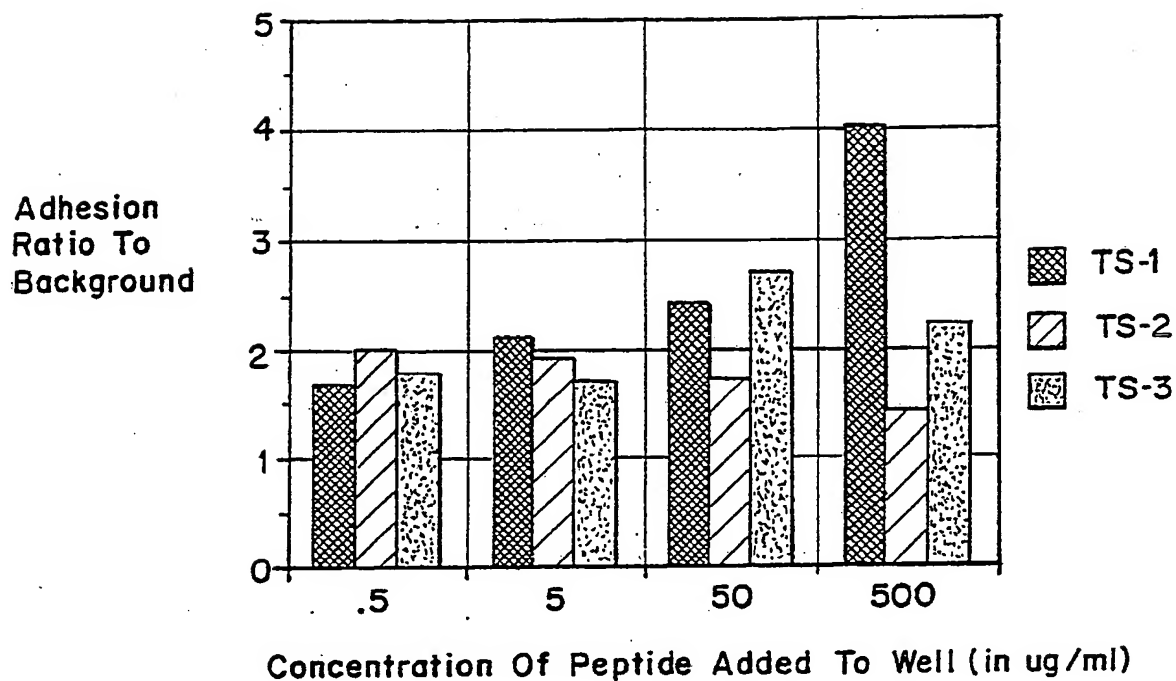
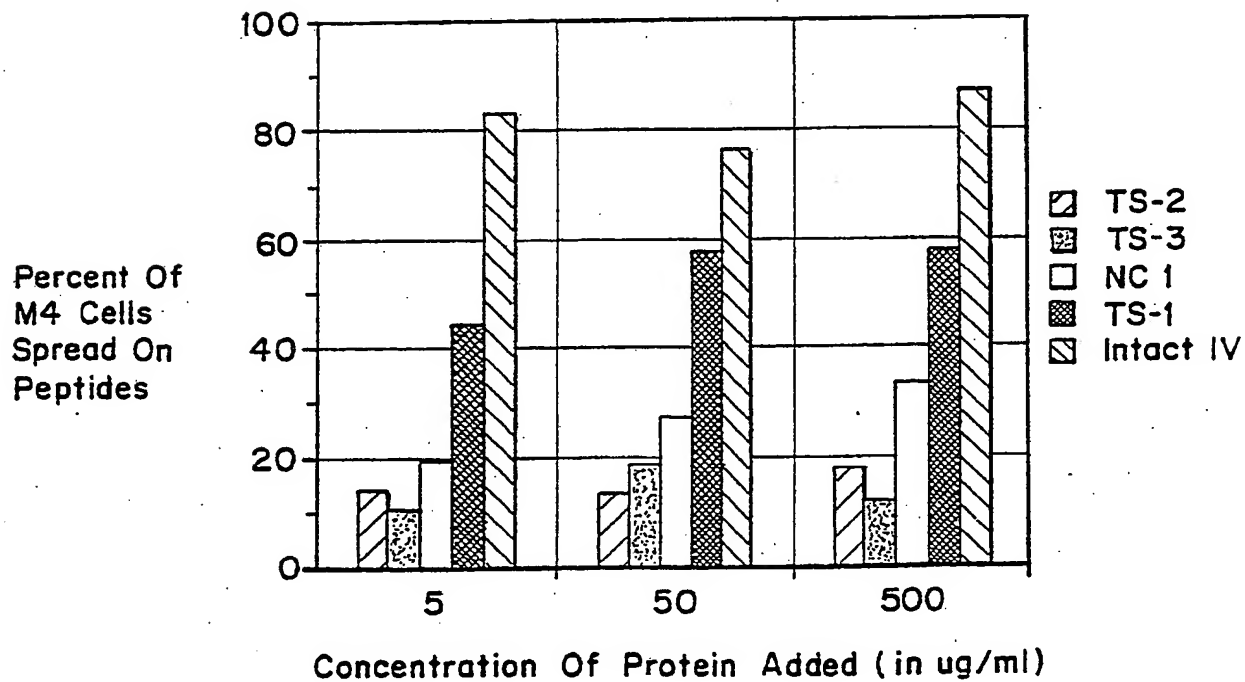


FIG. 16



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FIG. 17

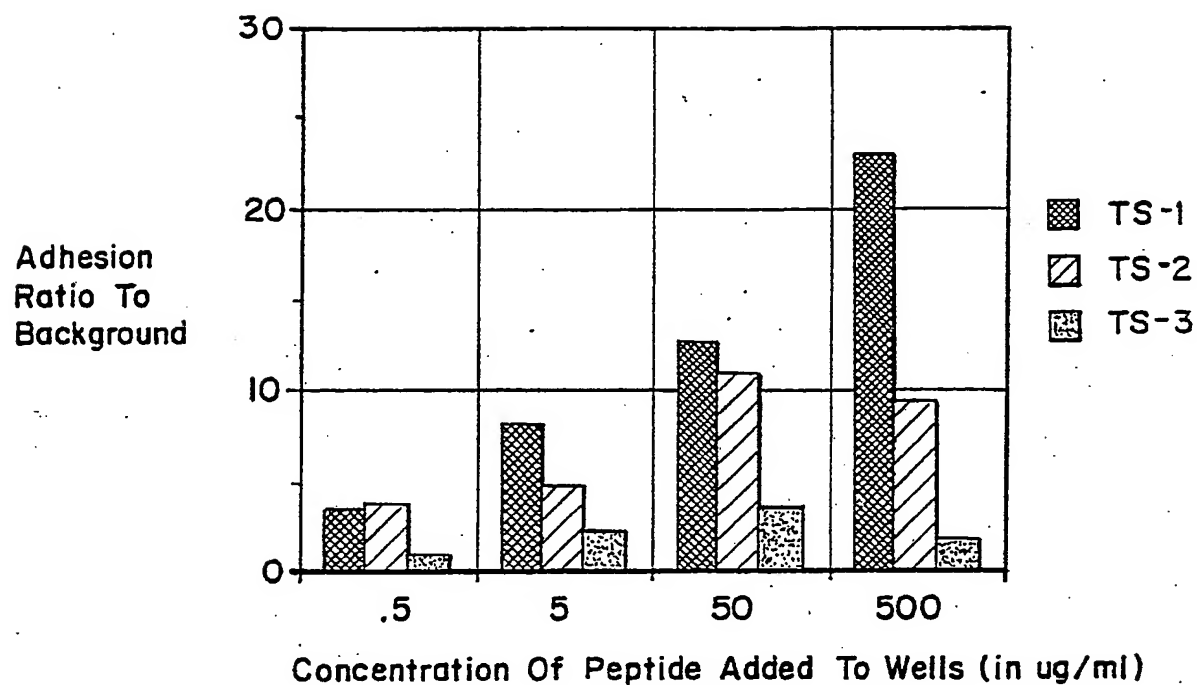
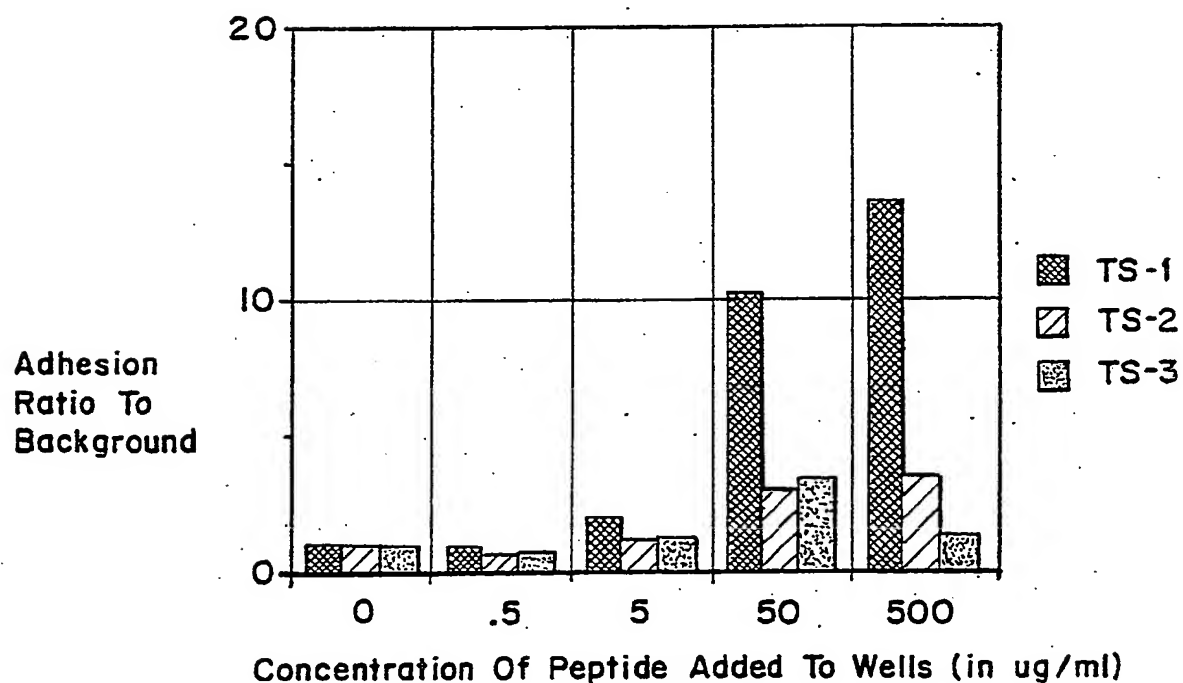


FIG. 18



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FIG. 19

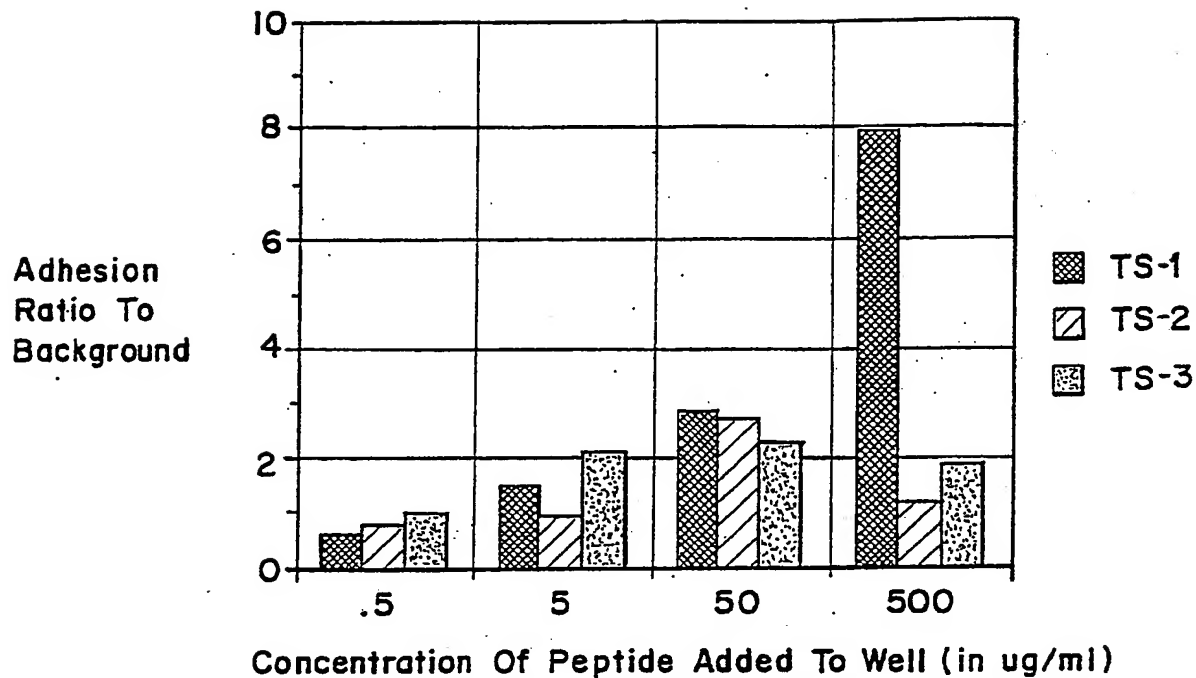
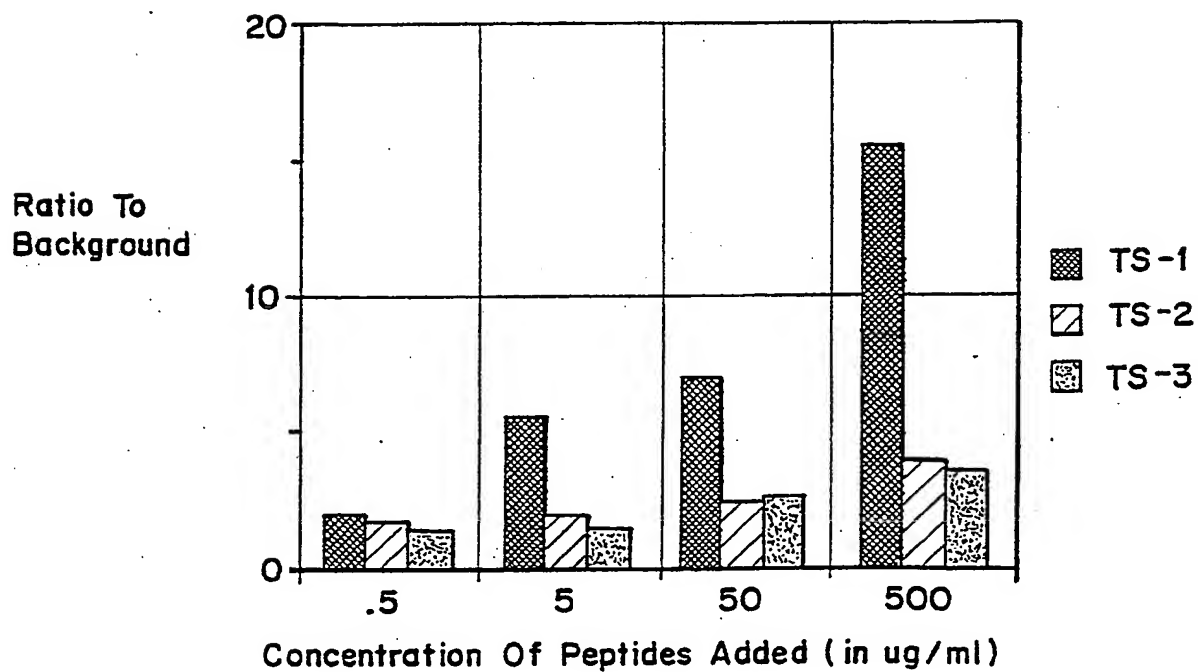
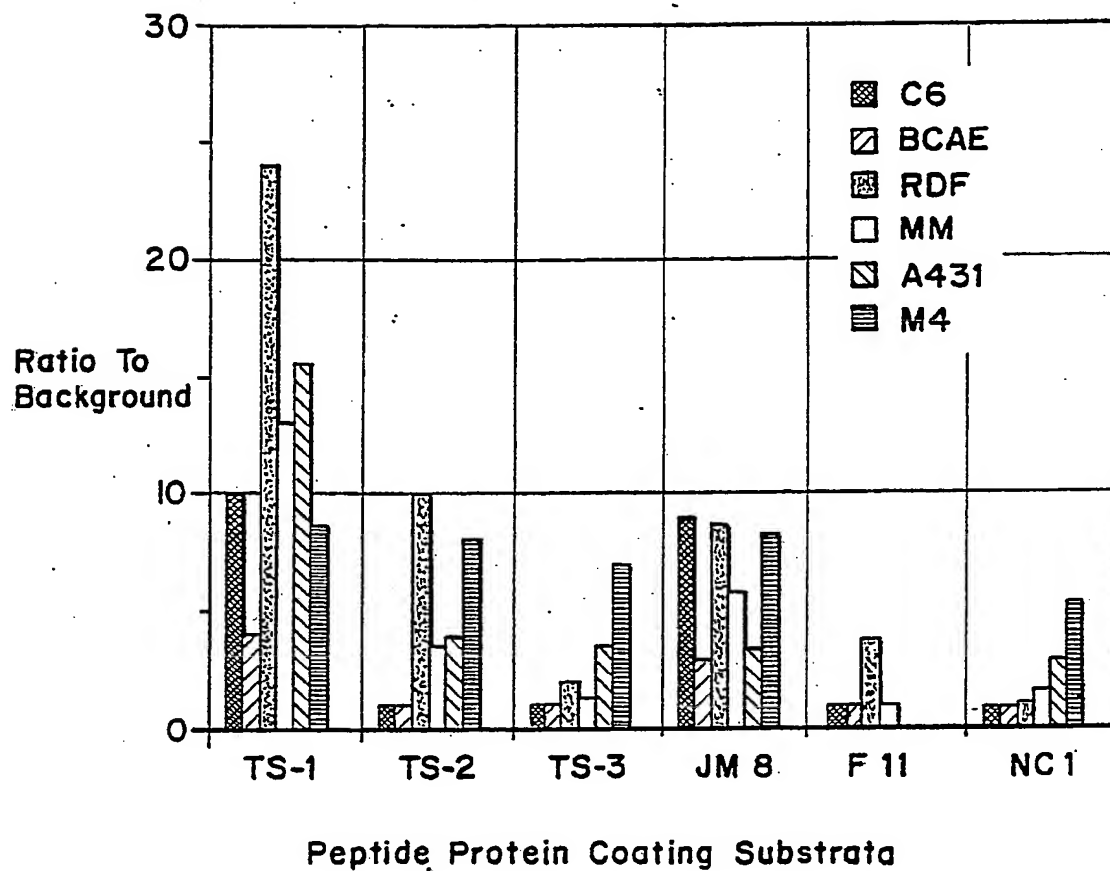


FIG. 20



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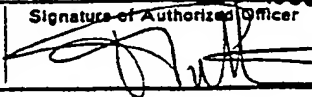
FIG. 21



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03023

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 07 K 7/08; C 07 K 7/10; A 61 F 2/02; A 61 L 27/00; A 61 L 15/01; C 12 M 3/04		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 07 K 7/00; A 61 F 2/00; A 61 L 27/00; A 61 L 15/00; C 12 M 3/00; A 61 K 37/00	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	EP, A, 0214035 (INST. MERIEUX) 11 March 1987 see page 11, claim 26 --	1-10, 16, 17
A	European Journal of Biochemistry, volume 147, no. 2, 1985, FEBS, I. Oberbäumer et al.: "Amino acid sequence of the non-collagenous globular domain (NC1) of the $\alpha 1$ (IV) chain of basement membrane collagen as derived from complementary DNA", pages 217-224 see page 221; figure 6 --	1
A	The Journal of Biological Chemistry, volume 260, no. 12, 25 June 1986, The American Society of Biological Chemists, Inc., (US), T. Pihlajaniemi et al.: "cDNA clones coding for the pro- $\alpha 1$ (IV) chain of ./."	1
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 18th November 1988	Date of Mailing of this International Search Report 12 DEC 1988	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer  P.C.G. VAN DER PUTTEN	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	<p>human type IV procollagen reveal an unusual homology of amino acid sequences in two halves of the carboxyl-terminal domain", pages 7681-7687 see page 7683; figure 2</p> <p>La Recherche, volume 14, no. 141, February 1983, (Paris, FR), C. Tutin: "Comment fabriquer des peaux artificielles", pages 252-254 see the whole document</p>	1,2,8-10
A ₂	<p>Chemical Abstracts, volume 101, 1984, (Columbus, Ohio, US), F. Misselwitz et al.: "Platelet adhesion and aggregation on surfaces coated with human collagens of type I, III, IV, and V", see page 442, abstract 208567b, & Byull. Eksp. Biol. Med. 1984, 98(9), 359-64</p>	1,11-15

US 8803023
SA 24513

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0214035	11-03-87	JP-A- 62053927 FR-A- 2586703	09-03-87 06-03-87

EPO For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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